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Priming of microbial microcystin degradation in biomass-fed gravity driven membrane filtration biofilms

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ABSTRACT

Gravity-driven membrane (GDM) filtration is a promising tool for low-cost decentralized drinking water production. The biofilms in GDM systems are able of removing harmful chemical components, particularly toxic cyanobacterial metabolites such as microcystins (MCs). This is relevant for the application of GDM filtration because anthropogenic nutrient input and climate change have led to an increase of toxic cyanobacterial blooms. However, removal of MCs in newly developing GDM biofilms is only established after a prolonged period of time. Since cyanobacterial blooms are transient phenomena, it is important to understand MC removal in mature biofilms with or without prior toxin exposure. In this study, the microbial community composition of GDM biofilms was investigated in systems fed with water from a lake with periodic blooms of MC-producing cyanobacteria. Two out of three experimental treatments were supplemented with dead biomass of a MC-containing cyanobacterial strain, or of a non-toxic mutant, respectively. Analysis of bacterial rRNA genes revealed that both biomass-amended treatments were significantly more similar to each other than to a non-supplemented control. Therefore, it was hypothesized that biofilms could potentially be 'primed' for rapid MC removal by prior addition of non-toxic biomass. A subsequent experiment showed that MC removal developed significantly faster in mature biofilms that were pre-fed with biomass from the mutant strain than in unamended controls, indicating that MC degradation was a facultative trait of bacterial populations in GDM biofilms. The significant enrichment of bacteria related to both aerobic and anaerobic MC degraders suggested that this process might have occurred in parallel in different microniches.

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Introduction

Harmful algal blooms (HABs) have become a global concern in many freshwater, estuarine and marine ecosystems [55]. Triggers for the rise of HABs are an interplay of climate change-related factors (e.g. increasing water temperature), or changes in water body stratification patterns, in combination with anthropogenic eutrophication [61]. Frequently, the dominant HAB-forming species are related to various genera of cyanobacteria, such as *Microcystis*, *Nodularia*, *Anabaena*, *Trichodesmium* and *Planktothrix* [55], which are all known producers of toxic secondary metabolites. Microcystins (MCs) are amongst the most common toxins produced by cyanobacteria in fresh and brackish water systems [70]. These cyclic peptides are classified as potent hepatotoxins, since they are taken up by hepatocytes where they inhibit the activity of eukaryotic protein phosphatase 1 and 2A [27]. Depending on the concentration and exposure time, MCs can cause acute [2] or chronic [25] poisoning. Exposure to sublethal MC concentrations induces hepatic oxidative stress [30], and some studies indicate that MCs promote liver tumors [36]. MC concentrations can rise to 1800 $\mu g\,L^{-1}$ during the collapse of HABs [70].

A number of bacteria have been described as MC degraders, such as *Sphingomonas* sp. [37], *Stenotrophomonas* sp. [10], *Pseudomonas aeruginosa* [75], *Microbacterium* sp. [63] and *Burkholderia* sp. [45]. While the details of the MC degradation pathway are not clear, several studies suggest that degradation might be carried out by intracellular enzymes of the *mlr* gene cluster [6,37]. Other studies indicate that other degradation pathways might exist [23,49].

Drinking water is the main source of MC uptake for humans and animals via oral ingestion [25]. The World Health Organization (WHO) has proposed an upper limit of 1 $\mu g\,L^{-1}$ for a common MC variant (MC-LR) in safe drinking water [81]. Conventional water treatment methods, such as coagulation, are effective at removing intact cyanobacterial cells but not the toxins that are dissolved or present in the fragments of broken cells [12]. Powdered activated carbon [13], slow sand filtration [28], reverse osmosis [51], and fil-

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tration membranes [26] are all potential means for MC removal. Chlorination [33] and ozonization [65] have also been suggested to be most effective for this purpose. Various combinations of these methods are commonly used in developed countries to provide safe drinking water that does not contain either cyanobacterial biomass or free cyanotoxins.

Community-scale processing technology may not always be available in developing and transient countries to treat surface waters during HABs. Gravity-driven membrane (GDM) filtration systems represent a decentralized alternative for producing drinking water on a small scale. In contrast to other membrane-based water purification approaches, biofilm formation is tolerated in the GDM system, and no pre- and/or post-treatment, chemical cleaning, backwashing or flushing is used. The GDM filtration setup is simple; it operates under ultra-low pressure in a dead-end mode, and GDM systems have been shown to be operationally stable for several months [59]. GDM filtration is a cost-effective technology for removing suspended organic and inorganic solids, pathogens and other disease vectors from raw water [59]. While the undisturbed biofilm formation is one of the main causes for initial flux decline in GDM systems, an equilibrium is eventually reached where the flux remains stable at a lower level over extended periods of time [17].

The biofilm of GDM filtration systems can improve permeate quality [11], and may delay microbial regrowth via the reduction of readily available organic carbon [16]. Moreover, biofilms were also shown to be effective against cyanotoxins: MCs in concentrations typically observed during HABs can be completely removed after an adaptation period for the biofilms of approximately 2 weeks [42]. Differences between the phylogenetic composition of bacteria in MC-treated biofilms and untreated controls have suggested that *Betaproteobacteria* might be particularly relevant for MC degradation. Other reports also point to bacteria from this class (*Burkholderia* sp. and *Methylobacillus* sp.) as potential MC degraders [35,45].

The communities described by Kohler et al. [42] were conspicuously similar in experimental treatments where MC-containing cyanobacteria were either added as live cells or as broken cell fragments. Thus, it is not clear if the MC-degradation capacity of these biofilms was indeed triggered by the presence of the toxin itself, or if the growth of facultative MC-degrading bacteria was favored by the richer conditions due to the addition of biomass. This difference might be of practical importance in the long-term use of GDM filtration systems. Specifically, it is conceivable that such systems might develop the potential for rapid toxin degradation even in the absence of HABs (e.g. triggered by prior input of biomass from non-toxic algal species).

The aim of this study was to explore the hypothesis that GDM biofilms would develop highly similar community composition when fed with biomass from either a toxic or a non-toxic strain of a cyanobacterial species (*Microcystis* sp.). In addition, another objective was to test if mature, biomass-amended biofilms with no prior exposure to MCs would exhibit a more rapid toxin degradation response than biofilms that had matured without biomass addition.

Materials and methods

Cyanobacterial cultures

Microcystis aeruginosa PCC 7806 (wild type) and a non-microcystin producing mutant strain [19] were grown in cyanobacterial growth medium [40] at 20 °C under constant light at 3.5 μmol quanta m^{-2} s^{-1}. Several Erlenmeyer flasks were inoculated with the wild type and the mutant strain in order to produce enough biomass for the experiments, and the cultures were grown for approximately 3 weeks. After this period, the cultures were harvested, divided into 50 mL tubes and stored frozen at −20 °C. For the experiments, these tubes were subjected to three freeze–thaw cycles in order to lyse the cyanobacterial cells, and to release the microcystins (MCs) from the wild-type strain. The absence of MCs in identically processed samples of the mutant strain was confirmed by HPLC–MS analysis (see below).

Experimental system

Water from Lake Zurich at a depth of 5 m was pumped into the gravity-driven membrane (GDM) filtration system by a centrifugal pump. The water was passed through three sedimentation tanks and was collected in a 40 L feed water tank (Fig. 1). The feed water was distributed to the biofouling monitors by silicon tubes. There was a syringe connection between the feed water tank and the biofouling monitors for spiking substrates into the system without direct contact with the feed water. A polyethersulfone ultrafiltration membrane with 150 kDa nominal cut-off (PBHK, Biomax Millipore, Billerica, MA, USA) was placed in each biofouling monitor. The biofouling monitors, the permeate collection bottles and their connections were all sterilized. The membrane was first soaked in 40% ethanol for 1.5 h and then placed in sterile deionized water overnight prior to the experiments [31]. The GDM system was operated under ultra-low pressure (65 mbar). The whole system, except for the permeate collection bottles, was kept in the dark and operated at room temperature.

Experimental procedure

Two experiments with a comparable setup were conducted, the first one in June 2014 and the second in March 2016. In experiment A (Exp A), three GDM filtration variants were set up in parallel: the unamended treatment only received lake water (LW), whereas the wild-type treatment (WT) was additionally supplied with MC-containing biomass from *M. aeruginosa*, and the mutant treatment (MUT) was supplied with the same amount of MC-free biomass from the mutant strain of *M. aeruginosa*. In experiment B (Exp B), the same three treatment types as in Exp A were set up initially in triplicates. However, once the WT treatment had reached 100% MC removal efficiency, the conditions in the MUT and LW treatments were changed by supplying them with the same amount of MC-containing biomass from *M. aeruginosa* that was added to the WT treatment (discontinuing the addition of biomass from the mutant strain in the MUT treatment). In both experiments, the corresponding biomass type was supplied every 24 h. No biomass was supplied and no data were collected from Exp B on days 21 and 22. The amount of dry cell weight supplemented to the WT and MUT treatments was chosen to mimic approximately an intense natural bloom situation (i.e. on average 15 mg day^{-1}) with no significant differences between the treatments and experiments. However, the microcystin content of this biomass differed between the experiments, since it was 18 μg microcystin L^{-1} day^{-1}, and 92 μg L^{-1} day^{-1} in Exps A and B, respectively. This difference in MC biomass quota was most likely related to the growth phase of cyanobacterial cells at the harvesting time point [79]. The duration of Exps A and B was 20 days and 39 days, respectively. The permeate flux was monitored every 24 h, and a sample for the quantification of MC concentrations was collected daily from the permeates. The transmembrane flux was calculated from the volume collected daily in the permeate collection bottles and the membrane area (0.00191 m^2). The biofilm thickness and structure was assessed at the end of the experiments using optical coherence tomography (OCT) (model 930 nm Spectral Domain, Thorlabs GmbH, Dachau, Germany). The mean thickness was determined by Matlab® (Math-

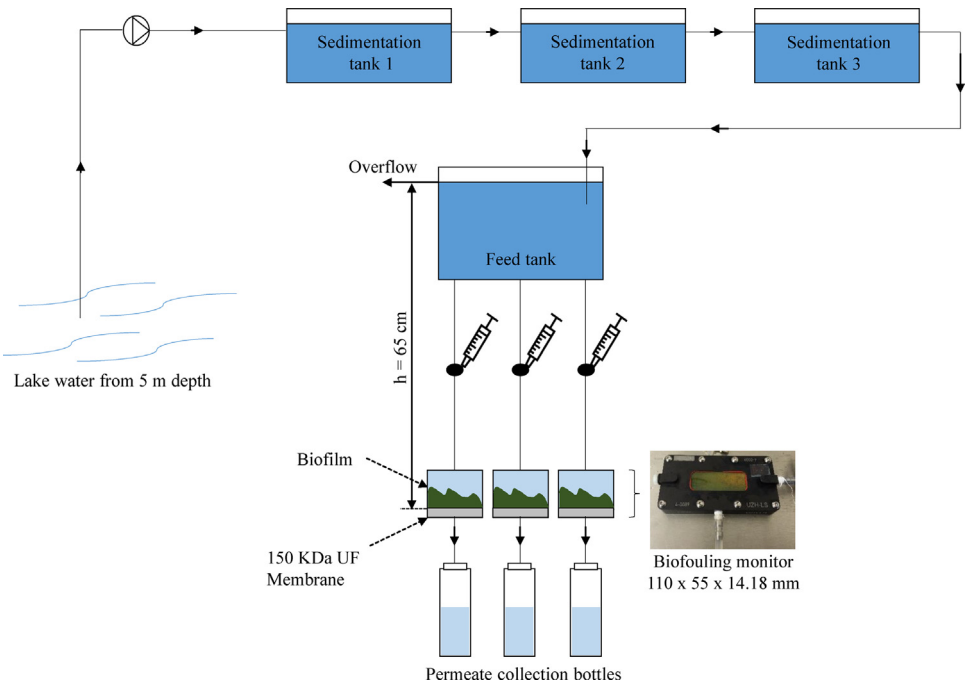

Fig. 1. Experimental setup of GDM filtration systems. UF: ultrafiltration.

Works, Natick, US), according to Derlon et al. [15]. The MC removal efficiency was calculated from the difference between the amount of MC added and the amount of toxin measured in the permeate collection bottles.

Microcystin quantification

Due to a change in instrumentation, the quantification of MCs in the two experiments was performed with different protocols and equipment. In Exp A, MCs were extracted from the total collected permeates by solid phase in a C18 cartridge (1 g, 60 mL, Mega Bond Elute, Varian, Agilent Technologies, Switzerland). Then, MCs were eluted in 100% methanol, dried in a vacuum rotary evaporator at 40 °C and 35 mbar, re-dissolved in 1 mL 60% methanol, filtered with a 0.2 μm polyethersulphone syringe filter (IC Acrodisc, Pall Corporation), and quantified as described in Kohler et al. [42] using high-performance liquid chromatography (HPLC) on a Shimadzu 10 AVP system equipped with a photodiode array detector and a C18 Hydrosphere column (YMC, 4.6 × 250 mm, Stagroma, Switzerland). Two microcystins from *M. aeruginosa* PCC7806, [D-Asp^3] MC-LR (*m*/*z* 981.5) and MC-LR (*m*/*z* 995.5) were extracted and purified (99%) through HPLC, and their specific molar absorption coefficient was used for preparing standard solutions in order to prepare a calibration curve. Quantification of the total MC concentration was achieved by determining the summed peak areas of each compound recorded at a wavelength of 239 nm.

In Exp B, 1 mL of sample was collected from the permeate collection bottles, filtered with a 0.2 μm polyethersulphone syringe filter (IC Acrodisc, Pall Corporation) and mixed with methanol to achieve a methanol concentration of 70%. MCs were directly analyzed on an HPLC system (1260 Infinity series, Agilent Technologies) and detected on an API 5000 triple quadrupole mass spectrometer (AB Sciex). The toxins were separated in a Synergi Fusion-RP C18 column (50 × 2.00 mm, 4 μm particle size, Phenomenex) with a constant column temperature of 60 °C. Solvent A was 1 mM formic acid (Fluka) in water and solvent B was 100% methanol (J.T. Baker, Avantor). The flow rate was maintained at 1 mL min^{-1}. The following gradient was applied: Solvent B from 20% to 80% for 5 min; B 100% for 2.5 min and B 20% for 2.5 min. Nodularin (5 μg L^{-1}) was used as the internal standard. The concentrations of MCs and nodularin (*m*/*z* 825.5) for the calibration curve and internal standards, respectively, were determined as described above. The software Analyst (version 1.6.1, AB Sciex) was used for the data acquisition and the MC quantification was carried out using the MultiQuant software (version 2.1, AB Sciex). The total MC concentration was calculated as described above.

DNA extraction, 16S rRNA sequencing and sequence processing

DNA extraction of all biofilms from the two experiments was performed using the UltraClean® Water DNA Isolation Kit (MO BIO Laboratories, Inc.), according to the manufacturer's specifications. The purified DNA was eluted in 10 mM Tris buffer and stored at −20 °C. Partial 16S rRNA gene sequences were obtained by next generation sequencing (NGS, Illumina MiSeq V3 subunit, 2 × 300 bp, LGC Genomics, Germany). The primer pair 799F-1115R was used to amplify the DNA of heterotrophic bacteria and exclude chloroplasts [9,64]. The reverse primer was modified by including a degenerate base (5′-AGGGTTGCGCTCGTTRC-3′) to increase the coverage against the current SILVA database (version 128) [62]. All samples were analyzed in technical replicates. Raw data were then processed by an in-house pipeline written in DELPHI, which included the joining of paired reads, dereplication, length trimming (250 bases), quality control (0.5 average error rate per sequence), chimera check, and clustering similar to the UPARSE algorithm [22]. Taxonomic assignment of operational taxonomic units (OTUs) was achieved by a pair-wise alignment (local alignment, Smith–Waterman algorithm, 99% cut-off similarity) of their representative sequences in the SILVA database.

The two data sets were normalized individually to the lowest total number of reads per sample using an R script [76]. The script rarefied each sample by removing one read from a randomly chosen OTU and this step was repeated until the total number of reads in the sample matched the number of reads in the smallest sample. The normalization was performed after OTU identification because OTUs are more robust if calculated on the complete sequence data set. Singletons were excluded after the normalization procedure.

Table 1
Exp A: ratios between genotypes (OTUs) that could be unambiguously classified as anaerobes or aerobes, respectively (using either OTU numbers or the number of reads represented by these OTU). The listing distinguishes between genotypes that were exclusively found in one treatment or shared between 2 or 3 treatments. Only mean values are given for the ratios of OTUs that were exclusive to a single treatment (N = 2). For the other (shared) OTUs, all possible pairwise combinations of biological replicates were considered for calculating the anaerobe/aerobe ratios (N = 4 and 12 for 2 and 3 treatments, respectively). Means and standard deviations of these combinations are given. Differences were assessed for significance by Kruskal–Wallis ANOVAs followed by Dunn's post-hoc tests.

	Occurrence	Anaerobic/aerobic	% of total OTUs/reads (anaerobic)	% of total OTUs/reads (aerobic)
OTUs	WT only	1.5	46	30
Reads		16.8	90	5
OTUs	MUT only	0.2	10	60
Reads		0.2	1	60
OTUs	LW only	0.08	3	40
Reads		0.03	2	70
OTUs	WT, MUT	0.6 (±0.08)	20 (±3)	40 (±4)
Reads		4.1 (±1.65)	70 (±11)	20 (±7)
OTUs	WT, LW	0.1 (±0.06)*	7 (±2)*	60 (±23)
Reads		0.1 (±0.11)*	7 (±5)**	60 (±13)
OTUs	MUT, LW	0.1 (±0.02)	7 (±1)*	50 (±1)
Reads		0.1 (±0.04)*	9 (±2)*	70 (±5)
OTUs	WT, MUT, LW	0.1 (±0.01)**	7 (±1)*	60 (±4)**
Reads		0.1 (±0.06)*	10 (±5)*	80 (±5)***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 2
List of 15 OTUs with highest read numbers in Exp B that were >99.2% similar to OTUs in Exp A. Except for the OTUs marked with asterisks, these OTUs were present in all treatments of Exp A. The fourth column indicates if the respective genotype had significantly higher read numbers in the WT and/or MUT treatments than in the LW treatment of Exp A. (1) Exclusively present in WT and MUT treatments of Exp A; (2) exclusively present in the WT treatment of Exp A. NA: Not assigned.

AccNr (% identity)	Relative abundance (Exp B, % of common OTUs)	Relative abundance (Exp A, % of common OTUs)	Δ relative abundance to LW (Exp A)	Related to known MC degraders	Metabolism	Taxonomy (% identity to the closest cultured relative)
AB473022 (100%)	19.5	<0.1	MUT	[48]	Facultative	*Aeromonas hydrophila* (99%)
JQ855508 (99.6%)	7.1	5.5	WT, MUT	[49]	Aerobic	*Variovorax*-to uncultured bacterium (*Variovorax paradoxus* 99%)
AF236013 (100%)	6.5	0.9	WT, MUT		Aerobic	*Undibacterium*-betaproteobacterium A1020 (*Undibacterium terreum* 96%)
AB576897 (100%)	6.0	0.3	MUT	[77]	Aerobic	*Pseudoxanthomonas*-uncultured bacterium (*Pseudoxanthomonas mexicana* 97%)
FM886906 (100%)	4.8	3.5	WT, MUT		Aerobic	*Mitsuaria chitosanitabida* (97%)
AB680727* (100%)	3.9	0.2	(1)		Aerobic	*Flavobacterium xanthum* (100%)
AY212651 (100%)	3.3	0.2	–	[24]	Aerobic	*Pseudomonas*-uncultured bacterium (*Pseudomonas benzenivorans* 97%)
HM141824 (100%)	3.0	1.9	WT, MUT		Anaerobic	*Blvii28* wastewater-sludge group-uncultured bacterium (*Parabacteroides distasonis* 83%)
DQ676400* (100%)	2.7	<0.1	(1)		NA	*Rhodocyclaceae*; uncultured-uncultured betaproteobacterium (*Ferribacterium limneticum* 96%)
AB672263 (100%)	2.6	3.6	WT, MUT		Aerobic	*Ideonella*-uncultured bacterium (*Ideonella dechloratans* 98%)
GU454906* (100%)	2.3	<0.1	(2)		Anaerobic	*Spirochaetaceae*; uncultured-uncultured bacterium (*Treponema stenostreptum* 90%)
AY212694 (100%)	2.2	0.1	–		Aerobic	*Aquabacterium*-uncultured bacterium (*Aquabacterium commune* 98%)
DQ469224* (100%)	2.2	<0.1	(1)	[71]	Aerobic	*Flavobacterium*-uncultured bacterium (*Flavobacterium succinicans* 99%)
AB672298 (100%)	1.9	<0.1	WT		Aerobic	*Zoogloea*-uncultured bacterium (*Zoogloea resiniphila* 97%)
JX262570 (99.6%)	1.6	0.6	WT, MUT	[38]	Anaerobic	*Acidaminobacter*-uncultured bacterium (*Acidaminobacter hydrogenoformans* 92%)

The Bray–Curtis dissimilarity index was calculated using the 'vegan' R package and default R functions (R version 3.3.2) [54] for each pair of samples. Since the technical replicates were not significantly different from each other in this analysis, they were pooled for subsequent analyses. Significant differences ($P < 0.05$) in the read numbers of OTUs between normalized samples were

established using the R package 'metagenomeSeq' [58]. Using published data for the closest cultivated relatives, an attempt was then made to classify the OTUs into genotypes with aerobic (facultative or obligate) or anaerobic metabolism. In total, 53% of all OTUs representing 91% reads could be unambiguously assigned to either category. Significant differences across the groups were calculated with the Kruskal–Wallis test and Dunn's post-hoc test with Holm's correction, using the 'dunn.test' in the R package [18] (Table 1). For the comparative analysis of the two experiments, a set of 'common OTUs' was defined by only including those OTUs from Exps A and B that were >99.2% identical (Table 2). Common OTUs represented 60% of the total number of reads from the normalized dataset for the two experiments. A data set was then produced that only included the reads from the common OTUs and the data were normalized to the sample with the lowest read number, as described above. Samples from this subset were clustered using Bray–Curtis dissimilarity and average linkage clustering (Fig. 5B). A similarity profile analysis ($P < 0.001$) identified 15 significantly different groups. The stability of the three major clusters was then tested by bootstrapping (1000 bootstrap interactions) using the 'clustsig' [80] and 'fpc' [32] R packages. The same procedure was also carried out on a normalized data set including all OTUs from Exps A and B (Fig. 5A).

Results

Experiment A

Physical and chemical parameters

The permeate flux in the LW treatments after the first 24 h period was 13 L m^{-2} h^{-1}. It then stabilized after 4 days operation and remained at approximately 10 L m^{-2} h^{-1}, resulting in a flux decrease of 23% (Fig. 2A). In the WT and MUT treatments, the permeate flux started at 9.5 L m^{-2} h^{-1} and tended to decrease throughout the experiment with no visible stabilization. Towards the end of the experiment, the permeate flux had declined by 82% and 75% in the WT and MUT treatments, respectively. The amended treatments (WT and MUT) had a 75% lower flux than the LW treatment. The thickness of the biofilms in the various treatments agreed with the respective flux patterns, in that it was <100 μm for the LW treatment, whereas the WT and MUT biofilms had a thickness in the range of 250 and 1000 μm.

The MC removal efficiency in the WT treatments started at 20% and reached 100% over the course of 15 days (Fig. 2B). Once the maximum MC removal efficiency was achieved it remained stable until the end of the experiment. The feed water was also monitored in terms of MC concentration but no free toxin was detected.

Microbial community diversity and composition

After normalization and removal of singletons, the microbial assemblages in the various Exp A treatments comprised 2331 OTUs. The WT treatments included 686 and 582 OTUs, the MUT 805 and 909 OTUs, and the LW 1569 and 1511 OTUs, compared to the two biological replicates, respectively. All the treatments had approximately the same number of sequence reads (95,000). The LW treatments featured the highest number of exclusive genotypes (Fig. 3), which represented 41% of the total number of OTUs, whereas substantially lower fractions of OTUs were specific to the WT and MUT treatments, respectively. In terms of reads, the exclusive OTUs from all three treatments together only represented 6.3% of the total read number. In contrast, 22% of all OTUs were shared between all treatments (subsequently termed the 'core' assemblage), and these shared genotypes also constituted the vast majority of reads (78%). The MUT and LW treatments also shared a comparatively high number of OTUs, although this only represented 3% of all reads. The WT and LW treatments shared both the lowest number of OTUs and reads of the three pairs. A total of 147 OTUs representing >10% of the total reads were exclusively present in the WT and MUT treatments. Pairwise comparisons within the core assemblage showed that only 8 OTUs significantly differed in terms of read numbers between the WT and MUT treatments, whereas there were more than 10 times as many OTUs that differed between the LW and the WT (108 OTUs) or the LW and MUT (120 OTUs) treatments, respectively. In the subset of 147 OTUs that were exclusive to the WT and MUT treatments, only nine differed significantly in read numbers.

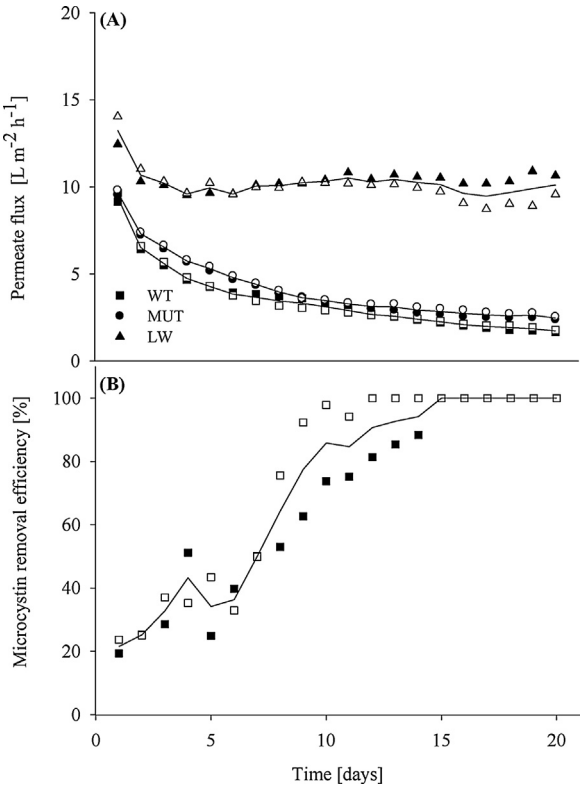

Fig. 2. Experiment A: (A) development of mean permeate flux. (B) Microcystin removal efficiency during 20 days operation (it only shows the WT treatment). The two biological replicates of each treatment are represented by black and white symbols and the mean by the solid line. Treatments: WT: addition of biomass from toxin-containing (wild type) *Microcystis* sp.; MUT: addition of biomass from toxin-free (mutant) *Microcystis* sp.; LW: fed with lake water without additional biomass.

Metabolic classification revealed that aerobic genotypes were generally more common, whereas anaerobes dominated in the subset of OTUs that were either exclusive to the WT or that only occurred in both the WT and MUT treatments (Table 1). The subcommunity shared between the two amended treatments (WT, MUT) featured approximately 30 times higher ratios of anaerobic to aerobic genotypes than all the other shared subcommunities or the core community. In addition, this ratio was >100 times higher for genotypes that were exclusive to the WT treatment than for those that were exclusive to either MUT or LW.

Experiment B

Physical and chemical parameters

The initial permeate flux in the LW treatment was approximately 25 L m^{-2} h^{-1}. It then decreased by 68% over the following 8 days and stabilized at approximately 8 L m^{-2} h^{-1} until day 23 (Fig. 4A). When MC-containing biomass was added to the LW, the flux decreased by 87% during the next 6 days compared with the initial quantity and it stabilized at 1 L m^{-2} h^{-1}. In the WT and MUT treatments, the permeate flux after the first 24 h was 5 and

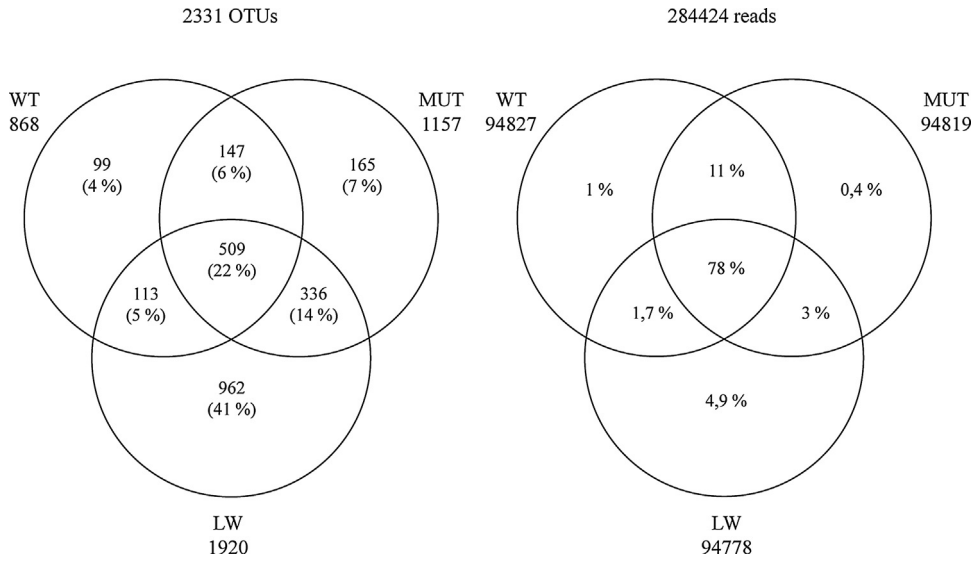

Fig. 3. Comparison of the microbial assemblages in the three treatments of Experiment A derived from 16S rRNA gene-defined operational taxonomic units (OTUs). The left and right sets of the Venn diagrams represent the proportions of shared OTUs and shared sequence reads, respectively.

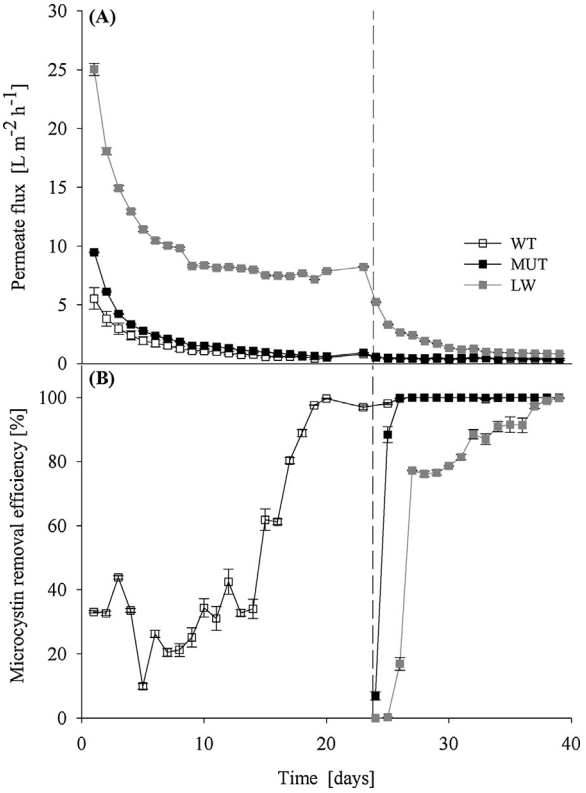

Fig. 4. Experiment B: (A) development of permeate flux in the different treatments (mean ± 1 SE, $n = 3$). (B) Microcystin removal efficiency for 39 days of GDM operation. The vertical line at day 23 represents the day when all treatments started to receive MC-containing biomass.

10 L m^{-2} h^{-1}, respectively. In these treatments, the flux stabilized at 0.5 L m^{-2} h^{-1} after 13 days operation, which corresponded to a reduction in permeability capacity of approximately 93%. The flux in the MUT treatment was not visibly affected by the change from MC-free to MC-containing biomass starting on day 23.

MC removal efficiency started at 33% and stayed at approximately this level until day 12, then it increased sharply and reached 100% after 20 days (Fig. 4B). The MUT treatment, which had been supplied with MC-free biomass until day 23, reached 100% of the MC removal efficiency after only 3 days exposure to MC-containing biomass. In contrast, the LW treatment required 14 more days to also reach the maximum MC removal efficiency.

Comparison of microbial community composition between experiments

Altogether, 1054 OTUs were found in the second experiment. Of these, the representative sequences of 614 OTUs were >99.2% identical to OTUs from Exp A. Cluster analysis of the common genotypes showed that there was a highly significant distinction between the LW treatments from Exp A, the biomass amended treatments (WT and MUT) from Exp A, and the three treatments from Exp B (Fig. 5B). Bootstrap analysis further confirmed the stability of these three clusters, and that the LW treatment of Exp A was separated significantly from the other two clusters. Similarity profile analyses confirmed that the respective technical replicates did not significantly differ from each other. In contrast, such differences were found for the biological replicates, which were, however, consistently more similar within than between treatments. The highest similarity was observed within the LW treatment from Exp B. Cluster and similarity profile analyses of the complete data set of all OTUs from Exps A and B resulted in the same pattern found for the common OTUs (Fig. 5A).

Since microbial community analysis in Exp B was performed after all the treatments had been fed with MC-containing cyanobacterial biomass for >2 weeks, the differences between the treatments were not assessed further. Instead, the study focused on the subset of OTUs from Exp B that were also present in Exp A. The 15 common OTUs with the highest read numbers in the common data set were then selected, which represented 70% and 50% of the total number of reads for the common OTUs and the complete data set of Exp B, respectively (Table 2). Of these top 15 common OTUs, nine were found to be significantly more abundant in the WT and MUT treatments of Exp A than in the LW, thereby unambiguously qualifying as universal 'responders' to biomass addition across the experiments. Moreover, six of the top 15 OTUs were closely affiliated (>99% similarity) to genotypes known for their ability to degrade MCs. The majority of these MC-degraders were classified as being aerobic, although two genotypes affiliated with the facultative and obligate anaerobic genera *Aeromonas* and *Acidaminobacter*, respectively.

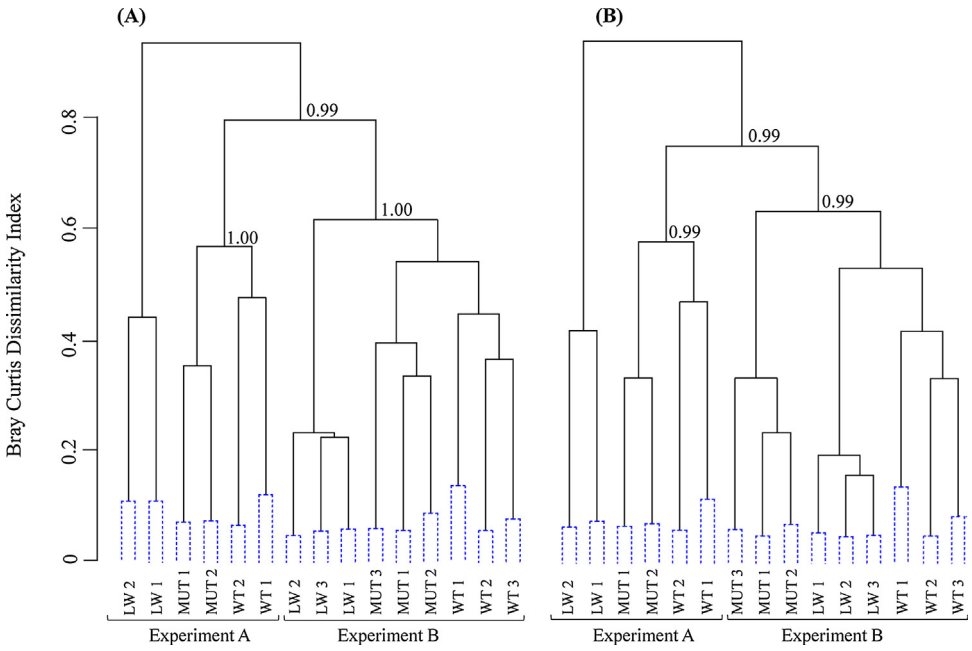

Fig. 5. Comparative analysis of Experiments A and B (Bray–Curtis dissimilarity), including (A) all genotypes and (B) only those genotypes that were present in both experiments. Similarity profile analysis identified 15 clusters that were significantly different from each other (solid lines, $P < 0.001$). No significant differences were found between technical replicates (dashed blue line). The stability of the three most deeply branching (i.e. most highly significant) clusters was tested by bootstrapping (indicated by the values at the branching points). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Discussion

Microbial diversity in GDM biofilms

Microbial biofilm communities are known to express different morphological and phenotypical characteristics than free living cells [74]. Moreover, there seems to be only a small genotypic overlap between genotypes that dominate in biofilms and in the water column of freshwater systems. Accordingly, bacteria from the typical planktonic lineages [52] were mostly absent from the GDM biofilms in this study. However, several genotypes in the samples with high read numbers have been previously described as colonizers of suspended organic particles (lake snow) [5], including the two largest OTUs in the 'core' community (i.e. genotypes that were universally present in all treatments), *Ideonella* and *Pelomonas* (*Comamonadaceae* family). These findings are in agreement with the proposition that biofilms may not only originate from single planktonic cells but also from the attachment of particles and cell aggregates [43]. Other important members of the GDM biofilms belonged to the *Comamonadaceae* family (*Variovorax* and *Acidovorax*) that are known as prominent components of similar habitats [3,47].

The 'core' community of the GDM biofilms (22% of OTUs; Fig. 3) represented the majority of all reads, implying that a large fraction of the community was shaped by the source rather than selected by the treatments. Previous experiments in a comparable setup [42] also suggested that the core community constituted over 50% of the total number of reads. Nevertheless, there were clear treatment-specific genotypic shifts in the bacterial assemblages even at low taxonomic resolution: *Alpha-*, *Betaproteobacteria* and *Bacteroidetes* were represented in similar proportions in the LW treatment, while *Betaproteobacteria* represented almost half of the genotypes in the amended treatments. Pronounced community shifts were also found in stream biofilms after enrichment with sucrose, nitrogen and phosphorus, but a subset of genotypes was shared between the control and the enriched treatments [78]. Moreover, bacterial diversity decreases in enriched stream biofilms [78], which agreed well with the observation from Exp A (Fig. 3).

Similarity between WT and MUT treatments in Exp A

Mature biofilms typically develop spatial heterogeneity, which in turn may create vertical niche separation [68,73]. In particular, steep oxygen gradients allow for the presence of strict anaerobes in the deeper strata of biofilms that form in fully aerated water [4,74]. In the GDM biofilms, numerous facultative anaerobic bacteria were identified, as well as various genotypes from strictly anaerobic lineages, such as *Ignavibacteriales*, *Clostridiales* or *Desulfovibrionales*. The vast majority (84%) of all anaerobic reads were found in the WT and MUT treatments, suggesting that the anaerobic lifestyle was a response to a more productive environment. Anaerobic zones and the corresponding genotypes are a common phenomenon in biofilms that develop under substrate-rich conditions in the aerated stages of wastewater treatment plants [53].

Both the biofilm thickness measurements and the permeate flux (Fig. 2A) indicated that the supplementation of biomass was responsible for the morphological and functional properties of biofilms irrespective of toxin content. The two biomass-amended treatments from Exp A, moreover, showed similar genotype ratios for anaerobes and aerobes (Table 1), likely reflecting a similar physical structure and community function for these biofilms. Genotypic community structure was generally very similar in these two treatments (Fig. 3). Moreover, only eight genotypes were found in the core community that were differentially expressed between WT and MUT, and nine such genotypes in the subset were exclusive to the WT and MUT treatments. A previous study on biofilm communities in GDM systems comparing the addition of intact (live) vs. ruptured *M. aeruginosa* cells also found highly similar communities in these treatments that differed from the unamended control [42].

Comparison of the two experiments

One incentive for repeating the experiment in a different season was to test if the addition of cyanobacterial biomass would

reproducibly select for a particular microbial subcommunity that differed from biofilm communities fed only with lake water. However, there were pronounced seasonal differences in the composition of the microbial assemblages of the source water [21]. Thus, the presence of season-specific genotypes might confound any systematic differences between biomass-amended and non-amended biofilms. Therefore, the community structure was compared using the complete data set (Fig. 5A), but additionally only including the genotypes that were common in the two experiments (approximately 22% of all OTUs or 60% of all reads).

Genotypic diversity in the two experiments was similar if the fraction of OTUs exclusive to the LW treatment in Exp A (962 OTUs) were taken into account (i.e. excluded from the comparison) (Exp A: 1369 OTUs; Exp B: 1054 OTUs). The dissimilarity analysis revealed that the biomass-enriched treatments of the two experiments were clearly separated (Fig. 5A). This was also the case even if only common OTUs were considered (Fig. 5B), possibly due to the influence of experiment-specific genotypes on their relative proportions (read numbers). At the same time, the most deeply branching separations in both the complete set of genotypes and in the common OTUs were between the communities from the non-amended LW treatment of Exp A and from treatments that had received cyanobacterial biomass. This indicated that the addition of biomass was the more important factor for determining the community structure of biofilms than seasonal differences in the composition of the source water.

Dissimilarity analysis also confirmed that the technical replicates were not significantly different, indicating that the sequencing effort was sufficient to obtain an unbiased view of individual GDM biofilm communities. Interestingly, this was not the case for biological replicates within identical treatments, since, although individual biological replicates were always most similar to others from the same treatment, they nevertheless had average Bray–Curtis dissimilarity scores between 40% and 50% (Fig. 5). A considerable number of non-singleton OTUs (19% and 12% in Exps A and B, respectively) were present in one biological replicate only. The assembly of local communities from a regional species pool is simultaneously driven by stochastic effects, such as immigration, drift and deterministic factors (i.e. 'environmental filtering' or 'species sorting') [44,46,50]. High diversity between local assemblages (i.e. beta diversity) might form in identical habitat conditions, for instance, due to priority effects that favor early arriving genotypes. Specifically, higher productivity appears to change community assembly processes towards stochasticity, resulting in greater beta diversity under comparable conditions [8]. This would be consistent with the findings from Exp A, considering only those genotypes that were specific for a particular treatment type, which showed the differences were higher between the various biomass-amended communities than between the ones fed with lake water only (Bray–Curtis dissimilarity indices of 0.78 ± 0.17 and 0.54, respectively). Moreover, Zhou et al. [84] demonstrated that even identical environmental conditions can lead to the formation of microbial communities with distinct genotypic composition that all originate from a single inoculum, and that these communities, moreover, differ in functional properties.

'Priming' of biofilm communities for a future function

The striking similarities between the microbial communities in the two biomass-amended treatments from Exp A led to the design and performance of a second experiment, to explore the notion that GDM biofilm assemblages could be prepared or 'primed' for toxin degradation even without previous exposure to high MC levels. It was hypothesized that, on addition of toxic cyanobacteria, a mature biofilm pre-fed with non-toxic biomass would achieve complete removal of MC faster than a corresponding biofilm that had formed with lake water only.

The outcome of Exp B supported the hypothesis, since the MUT treatment only required 3 days to achieve 100% MC removal efficiency after being supplemented with toxic biomass (Fig. 4B). In contrast, the same level of performance was only reached after 14 days by the biofilms of the LW treatment. Biofilms from both treatments had been grown previously for a period of two weeks, suggesting that biofilm 'maturation' in itself was not the key factor for the establishment of (potentially slow growing) MC degraders, but this was due rather to the specific growth conditions induced by the additional biomass. The 10 times higher ratios of anaerobic to aerobic genotypes in the MUT compared to the LW treatment (Exp A; Table 1) also suggested that the overall growth conditions in these biofilms were significantly altered, providing an appropriate environment for the establishment of both aerobic and anaerobic MC degraders (Table 2).

The addition of non-toxic biomass was thus sufficient for the MUT treatments to develop the potential for a significantly more rapid degradation of MCs. Therefore, it is proposed that this 'cryptic' feature might fall in the category of so-called 'priming' effects [29], which is a facilitation of the degradation of recalcitrant compounds by the concomitant availability of readily assimilable organic substrates or nutrients. Currently, there are only a few studies that explore the potential of this type of priming in aquatic systems. Carlson et al. [7] performed incubation experiments with water from two different stations in the Sargasso Sea after addition of surplus carbon, nitrogen and/or phosphorus. At the end of the incubations, the total organic carbon was lowest in treatments that had been amended with all three substrates and nutrients used. Since the overall carbon balance in these treatments was negative, the authors interpreted their findings in terms of a 'priming' effect. The system in the present study clearly differed from the one described above, since the degradation of MC was probably not directly stimulated by the presence of other, labile organic compounds. Instead, the rich conditions in the MUT treatments likely 'primed' the prior growth of facultative MC degraders that were well prepared to perform this function once exposed to the toxin. Therefore, it is proposed to expand the concept of 'priming' from a short-term, ecophysiological perspective to one that also encompasses historical aspects of community composition, in other words, the establishment of microbial populations relevant for a specific future function (e.g. toxin degradation) as a consequence of the previously prevailing growth conditions.

The potential for the degradation of xenobiotica is a known 'cryptic' feature of soil microbial communities, and Johnsen and Karlson [39] studied the degradation capacity for polycyclic aromatic hydrocarbons (PAHs) by bacteria from different soils, ranging from pristine to heavily contaminated. Pristine soils exposed to low background concentrations of PAHs were equally able to degrade the PAHs as the contaminated ones, supporting the idea that bacterial communities might maintain populations capable of metabolizing such compounds even when they are only present in trace amounts. The community scenario of the other degradation study differed from the one reported here in that the GDM biofilm assemblages were only formed during the experiment itself. Moreover, the differences in MC degradation between the MUT and LW treatments argues against low background concentrations of toxin in the source water (as deduced from the presence of MC-containing cyanobacteria in Lake Zurich [61]) acting as a trigger for the 'priming' effect. Instead, the results clearly indicated that MC degradation in the GDM biofilms was a facultative feature of populations with greater metabolic flexibility that became established preferably under richer growth conditions. Known facultative MC degraders include *P. aeruginosa* [75] and *Burkholderia* sp. [45].

Responders to season and biomass: potential MC degraders

Thirteen of the top 15 common genotypes from the two experiments were significantly enriched in (or exclusive to) the biomass-amended treatments of Exp A (Table 2). Several of these taxa are known to occur in engineered aquatic environments, for instance, *Undibacterium* spp. [41] are typical in drinking water biofilms, and *Zoogloea* [66] and members of the *Blvii28* wastewater-sludge group [69] are found in waste water treatment systems. Five of the genotypes in Table 2 were represented by very low read numbers in Exp A (<0.1%), and their respective proportions increased by >10 fold in all the Exp B treatments. These genotypes might be regarded as seasonally specific responders to biomass and/or MC addition. Three more genotypes also substantially increased their relative abundances in Exp B compared to Exp A, where they only formed between 0.3% and 0.9% of the reads in the biomass-amended communities, and significantly less in the LW treatment. While this may still hint at seasonal effects, it might also reflect the intrinsic variability of community assembly between independent experiments, or at best the semi-quantitative nature of community analysis based on PCR amplification of rRNA sequences. Finally, four genotypes in Table 2 had high read numbers in both experiments, and were significantly less frequent if no cyanobacterial biomass was added (Exp A, LW treatment). Thus, they specifically responded to the treatment irrespective of the season.

In the absence of additional information (e.g. from pure cultures [56], stable isotope or metagenomic analysis [49]), it is not possible to unambiguously identify these bacterial populations that were responsible for MC degradation in the GDM biofilms. Nevertheless, the comparison of the common genotypes from Exp A to Exp B with data from the published literature of known MC-degraders may help to reduce the list of candidates that might have been responsible for this function. Unfortunately, the genotypes that have not yet been identified as MC degraders will be missed by such an approach. Moreover, microbial communities in natural and engineered environments are often characterized by a high degree of functional redundancy [1]. Therefore, it is conceivable that an overlapping set of taxa was responsible for MC degradation in the two experiments, since such bacteria were found within both subsets of genotypes and were either seasonally or constitutively favored by the addition of cyanobacterial biomass. This is also suggested by the available data from the literature, since close relatives of six of the 15 most common genotypes in Table 2 are known MC degraders. The two genotypes with highest read numbers in Table 2 were related to *Aeromonas hydrophila* (only abundant in Exp B), which is a common facultative anaerobe in aquatic habitats [14], and *Variovorax paradoxus* (abundant in both experiments), which is an aerobe found in both soil and aquatic systems [67]. Both species are known degraders of various complex compounds [57,72], including cyanotoxins: *Aeromonas* spp. were recently reported to degrade MC-LR [48] and cylindrospermopsin [20], and metagenomics information from Lake Erie indicated a potential for MC degradation in *Variovorax* spp. [49]. Other genera in Table 2 with known MC degraders were *Pseudoxanthomonas* [77], *Pseudomonas* [24], *Flavobacterium* [71] and *Acidaminobacter* [38]. It is also noteworthy that two of the genera in Table 2 that harbor MC degraders featured a facultative (*Aeromonas*) or obligate (*Acidaminobacter*) anaerobic metabolism. Little is known about the biochemistry of anaerobic MC degradation, even though a number of microbial strains capable of this metabolism have been described (e.g. *Clostridium* [83], *Microbacterium* and *Rhizobium* [63], *Aeromonas* [48], and *Acidaminobacter* [38]). In addition, there is growing field evidence that this process may be relevant in the sediments of lakes affected by *Microcystis* blooms [34,82]. In view of the strong indication of anoxic niches in the biomass-amended biofilms (i.e. the proportional increase of reads and genotypes with anaerobic metabolism; Table 1), it is conceivable that the degradation of MCs in the GDM biofilms was performed in parallel by subcommunities of aerobic and anaerobic bacteria.

In summary, this study added another aspect to the understanding of the beneficial properties of biofilms on the quality of filtrates from GDM systems [42,60]. The results suggested that the GDM biofilm communities might develop potential for the degradation of cyanotoxins even from prior exposure to a bloom of non-toxic cyanobacteria. This may provide 'insurance' against MC accumulation in the filtrates if GDM systems are used for drinking water production from sources with infrequent blooms of MC-containing phytoplankton. It remains to be studied if other types of biomass (e.g. blooms of other algal species) would be sufficient to induce comparable 'priming' of GDM biofilm communities for MC degradation.

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